AWARD NUMBER: W81XWH-15-1-0161

TITLE: Gene-Specific Demethylation as Targeted Therapy in MDS

PRINCIPAL INVESTIGATOR: Daniel G. Tenen, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Boston
Boston, MA 02215-5491

REPORT DATE: July 2017

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
JULY 2017	Annual	15JUN2016-14JUN2017

4. TITLE AND SUBTITLE
Gene-Specific Demethylation as Targeted Therapy in MDS

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0161

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Daniel G. Tenen, MD

Email: dtenen@bidmc.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
330 Brookline Avenue
Boston, MA
02215-5491

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	8	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. INTRODUCTION:

Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. We have found that DNMT1 binds to RNA with stronger affinity than DNA of the same primary structure. This interaction inhibits DNMT1 enzymatic activity thereby preventing DNA methylation and the resultant silencing of the corresponding DiR-regulated gene loci. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

2. KEYWORDS:

Myelodysplastic syndrome; p15, DNA methylation; RNA

3. ACCOMPLISHMENTS:

What were the major goals of the project?

.

In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR*

What was accomplished under these goals?

Aim 1. *To reduce P15 locus specific genomic methylation by induction of its respective DiR*

Our previous studies demonstrated that downregulation of ecCEBPA led to decreased CEBPA mRNA and increased DNA methylation levels, whereas ectopic expression of ecCEBPA resulted in an opposite outcome. Further, we demonstrated that RNA has a stronger affinity than DNA to DNMT1 and that RNA specifically interacts with the DNMT1 catalytic domain, leading to the hypothesis that RNA oligonucleotides could be utilized as gene specific demethylating agents. We continued exploring this avenue and chose as a model for this study two tumor suppressor genes frequently methylated in cancer: the CDKN2A (aka P16) and CDKN2B (aka: P15). While P16 is commonly methylated in solid tumors, P15 is silenced in myeloid disorders. Therefore, we decided to apply the same RNA-mediated demethylating approach to both genes. During the second funded year, we have established and optimized the conditions to perform RNA- and DNA- Fluorescence In Situ

Hybridization (FISH) for P15. Given that both techniques do not require a large number of cells, we will be able to apply this procedure on MDS primary samples, not only to assess the integrity of the locus, which is often deleted in cancer, but also to visualize the presence of P15 DiRs and their induction and/or upregulation upon DiR-mimicking oligonucleotides delivery.

In parallel, we have begun to collecting primary MDS samples in which we plan to evaluate the presence of natural existing DiRs. For this purpose, we are performing a full characterization of the P15 locus, mapping by 5'-3' RACE and localizing by FISH, the gene and the respective DiR-like transcripts on P15–expressing cell lines. During this year, we have been evaluating the contributions of different transcripts arising from the P15 locus, including the antisense non-coding RNA in the INK4 locus (ANRIL), a spliced long non-coding RNA consisting of 19 exons and located within the P15/P16/P14 gene cluster, whose function still remains to be elucidated. We applied a Click-iT® technology and combined it with deep sequencing of nuclear RNAs to identify all the potential transcripts with features similar to ecCEBPA (enriched in the nucleus and transcribed during the S-phase), including those corresponding to the P15 and P16 loci. Briefly, we performed nuclear RNA isolation, RNA purification, high-throughput sequencing, and combined this approach with the Click-iT® technology that is based on the biorthogonal click chemistry reaction, which enables the metabolic incorporation of ethynyl uridine (EU), a "clickable" ribonucleoside, into RNA during nascent RNA synthesis. Thus, biotin is "clicked" onto the nascent chain and streptavidin magnetic beads capture all newly synthesized transcripts. To compare the transcriptional profiles under these conditions with our previous results, we performed RNA-seq on total and nuclear RNA fractions of unsynchronized and S-phase synchronized HL-60 cells. Although HL-60 lacks both P16/P15 proteins, the entire locus displayed antisense transcription of ANRIL, a long non-coding RNA antisense to P15 reported as a P15 silencer. ANRIL acquires the highest levels of expression during the S-phase in the nuclear compartment, pointing to a potential counteracting effect on the DiR-like transcripts arising from the P15 locus. In line with these results, we observed that ANRIL precedes the expression of P15 mRNA during the S phase, peaking two hours after the release into the S phase, whereas P15 mRNA reaches its highest expression six hours after the release. We are now designing a mapping strategy to characterize both expressing and not-expressing cell lines.

Overall, these results suggest a complex regulation of the locus that can be dictated by a fine-tuning of sense and antisense transcription and enhance the relevance of RNA as a therapeutic tool to control gene expression.

Aim 2. *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

The goal of this aim is to evaluate whether RNA can be repurposed as a gene-specific demethylating tool to correct aberrant DNA methylation. P15 is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. This past year, our laboratory has been testing a double stranded RNA platform, the short activating RNAs (saRNAs) to reactivate P15 gene expression. saRNAs are small double stranded RNAs that were shown to induce gene expression in a gene-specific manner, yet the mechanism behind this reactivation remains unknown. Our hypothesis is that the saRNAs might be acting as DiRs-mimicking molecules, and we will investigate whether saRNAs induce demethylation of targeted loci. Thus, in collaboration with the U.K. biotech firm MiNA Therapeutics (for further information please refer to the OUTREACH Study: https://clinicaltrials.gov/ct2/show/NCT02716012), three saRNAs targeting different genomic locations within the P15 locus were designed: saP15-313 (promoter); saP15-11 (first- exon); and saP15-56 (intron). All three saRNAs were delivered into the myeloid cell line KG1a, which is heavily methylated at the P15 locus. Seventy-two hours after transfection, we observed reactivation of P15 expression as compared to a scrambled control, for one out of the three designed saRNAs, the one located in the first exon (PR11) and coinciding with the CpG island frequently methylated in MDS and other cancers. We repeatedly observed this effect in both synchronized and unsynchronized cells. Prompted by these results, we have analyzed the methylation changes in the P15 locus by DNA methylation specific PCR (MSP) and Combined Bisulfite Restriction Analysis (COBRA). This initial screening revealed an effect on DNA methylation, with acquisition of an unmethylated band by MSP and loss of the digestion pattern by COBRA. Therefore, we have designed and optimized a

bisulfite sequencing approach to quantitatively analyze the methylation changes within the region spanning the promoter and the first exon (between -280 and +802 bp from the transcription start site of P15), and we are assaying by this method the saPR11 changes on the methylation profile. To assess the minimum number of transcripts driving the locus demethylation, we are generating an array of standard curves to measure both gene reactivation and DiR-like transcripts, based on copy number quantitation. This strategy allows us to obtain an accurate quantification of the transcriptional activity not only on the bulk cell population, but also at single cell resolution. In parallel, by RNA FISH, we can monitor the RNA induction and correlate the imaging sensitivity with the copy number changes. Provided that all the listed techniques do not require a large amount of cell number, we can translate this strategy to freshly collected primary MDS samples. Because saRNAs have been shown to be more effective with longer treatments, perhaps due to nuclear localization of the double stranded RNAs, our future plan is to prolong treatment with saPR-11, and analyze the effects at different time points. Further, to account for off-target effects of gene-specific versus non-specific demethylation approaches, we will compare the impact of saPR-11 treatment to the clinically approved non-specific demethylating agent 5-Azacytidine treatment using a genome-wide method as previously described (Di Ruscio et al, Nature 503:371, 2013).

What opportunities for training and professional development has the project provided? Nothing to report

How were the results disseminated to communities of interest? Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

a) We will continue to characterize the DiRs in the p15 and p16 loci, including mapping their 5' and 3' ends, not only in cell lines but in primary MDS patient samples
b) We will design and produce lentiviral particles for expression of specific p15 and p16 DiRs, and initiate experiments to express the first in cell lines, followed by expression in MDS patients samples in which these genes are not expressed in methylated.
c) We will assess the ability of these DiRs to induce demethylation and expression of P15 and P16.
d) We will continue to develop methods to visualize these RNAs by RNA-FISH
e) We will continue to develop oligonucleotides which mimic the action of the DiRs, both triplex forming oligonucleotides (TFOs) and chimeric RNA oligonucleotides (CROs).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project? Nothing to report

What was the impact on other disciplines? Nothing to report

What was the impact on technology transfer? Nothing to report

What was the impact on society beyond science and technology? Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

Changes in approach and reasons for change: Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to report

Changes that had a significant impact on expenditures: Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report

Significant changes in use or care of human subjects: Nothing to report

Significant changes in use or care of vertebrate animals: Nothing to report

Significant changes in use of biohazards and/or select agents: Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  Books or other non-periodical, one-time publications.

  Other publications, conference papers and presentations..

- **Website(s) or other Internet site(s)**

- **Technologies or techniques**

- **Inventions, patent applications, and/or licenses**

- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project? No changes

Name:	Daniel G. Tenen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6423-3888
Nearest person month worked:	2.4
Contribution to Project:	Dr. Tenen is overseeing the direction of the project
Funding Support:	This grant and other NIH grants

Name	Junyan Zhang
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms Zhang assists Dr. Tenen and other postdocs in this project
Funding Support:	This grant and other NIH grants

Name	Anais Wanet, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Dr. Wanet is focusing on Aim 1
Funding Support:	This grant and other NIH grants

Name	Mee Rie Sheen, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.77
Contribution to Project:	Dr. Sheen is focusing on Aim 2
Funding Support:	This grant and other NIH grants

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

What other organizations were involved as partners? None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not Applicable

9. APPENDICES: None